IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Oreste, *et al.*
Serial No.: 10/582,687
Filed: June 13, 2006
Title: LOW MOLECULAR WEIGHT POLYSACCHARIDES HAVING ANTITHROMBOTIC ACTIVITY

Group Art Unit: 1623
Examiner: Layla D. Bland

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

I, Pasqua ORESTE, a biochemist graduated in Biology at the University of Milan (Italy) do hereby declare as follows:

1. I am joint inventor of the instant application and jointly invented the whole subject matter claimed therein together with the other inventor, Giorgio Zoppetti. I am familiar with the instant application and have read the Office Action mailed on March 19, 2009 as well as the cited references that I know very well.
2. I am also familiar with literatures regarding polysaccharide K5 and its semisynthetic derivatives, in particular with the Oreste et al. application published as WO 02/50125 cited by the Examiner. In addition, I am one of the inventors and jointly invented with the other inventor, Giorgio Zoppetti, the whole subject matter disclosed therein.
3. The Oreste et al. reference discloses epiK5-N,O-sulfates having high antithrombin activity, and low molecular weight (LMW) epiK5-N,O-sulfates, derived therefrom by depolymerization, still having a very high antithrombin activity, by far higher than that of heparin.
4. The other inventor and I (herein below the term "we" will be used to indicate that the other inventor and I made all the intellectual and practical work together) put into practice the early depolymerization in order to try an alternative route to similar depolymerized products possibly overcoming the drawback of a hardly reproducible activity of said similar produts obtained by depolymerization at the end of the process of the Oreste et al. reference.

5. We did not even imagine that, by using the same process starting from the tertiary or quaternary salt of a depolymerized LMW-epiK5-N-sulfate, we would discover new LMW-epiK5-N,O-sulfates having not only low antithrombin activity, i.e. opposite to the corresponding activity of the LMW-epiK5-N,O-sulfates of the Oreste et al. reference, but also showing an antithrombotic profile qualitatively similar to that of low molecular heparin (LMWH).

6. The sole explanation of this surprising finding is that the unexpected profile of activity of the obtained products is due to the high content in 3-O-sulfate groups in the glucosamine units ("A-3-OS groups"), unless the presence of sulfate groups in the 2,5-anhydromannitol end unit also plays a non-negligible role. Such a possible influence of the sulfated 2,5-anhydromannitol end units would be even more surprising because it is known that, in the case of heparin, the activity of depolymerized LMWH is independent of its preparation method or, in other words, of the reducing or non reducing end units (Østergaard et al., Thrombosis Research, 1987, 45, 739-749, copy attached).

7. I do not - and any expert in glycosaminoglycan chemistry would certainly not - believe that this unexpected profile depends on the sulfation degree and this is proven by the Oreste et al. reference itself wherein the described products have a sulfation degree in the same range as those of the present application. The described process provides an oversulfation which, as it has been shown, introduces a great number of A-3-OS groups, and also provides an O-desulfation followed by a selective 6-O-sulfation and N-sulfation. As any expert in the heparin-like glycosaminoglycan field knows, as the cited prior art explicitly teaches, and as we duly submitted in the response to the previous OA, the A-3-OS groups are the last one to be desulfated during the desulfation step. By consequence, by modulating the O-desulfation time, the sulfation degree may be modulated without appreciable loss of A-3-OS groups.

8. The fact that the qualitative anticoagulant/antithrombotic activity of the products of claims 17-27 does not depend on the sulfation degree but on other factors, as set forth in item 6 above, is proven by an experiment made by myself or under my supervision. In 2006, our licensee, on behalf of a possible sub-licensee, asked me to provide said possible sub-licensee with a LMW-epiK5-N,O-sulfate according to the present invention in confidence. On the basis of our preceding experience (see Oreste et al.), we expected that the best activity would be obtained with a product having an average molecular weight higher than 7,000. Thus I prepared or supervised the preparation of said LMW-epiK5-N,O-sulfate which showed an average molecular weight of about 10,000 and a sulfation degree of 2.75. The product thus obtained showed an activity exactly overlapping that of the product of Example 1, but said activity was

not as high as I expected. Then, I was asked to lower the molecular weight to reach a molecular weight of about 6,000 as that of the product of Example 1 by depolymerization. The product thus obtained had a molecular weight of 6,000 and a sulfation degree of 2.32. Notwithstanding the loss of sulfate groups (but not in the 3-position of glucosamine) due to the acidic conditions of the depolymerization medium, said product showed Anti-Xa and Anti-IIa activities qualitatively similar to those of the parent product and of the product of Example 1 but with an Anti-Xa activity at a higher degree. The depolymerization step was reproduced twice under similar conditions with substantially identical results.

EXPERIMENTAL

Preparation of a LMW-epiK5-N-sulfate

(i) Epimerization to epiK5-N-sulfate

An amount of 2 g of K5 N-sulfate obtained as described in Example 2, steps (i) and (ii) of WO 02/068477 was dissolved in 60 ml of 25 mM HEPES buffer pH 7 containing 50 mM $CaCl_2$. The solution was recirculated through a 50 ml column containing 20 mg of immobilized recombinant C5-epimerase. The reaction was performed at 30°C with a flow rate of 40 ml/h for 24 hours. The product was purified by ultrafiltration and then precipitated with 3 volumes of ethanol. 1.8 g of epi-K5-N-sulfate having a 46.6% content in iduronic acid was obtained.

(ii) Depolymerization of epiK5-N-sulfate

An amount of 500 mg of epi K5 N-sulfate was dissolved in 10 ml of deionized water and the pH was measured (pH 6.8). The sample was cooled to 4°C and the pH was brought to 2.2 with a solution of 2% HCl. 8.97 mg of $NaNO_2$ (corresponding to 9 ml of a solution of 0.013 M $NaNO_2$ in water) were added and the reaction was left at 4°C for 20 minutes under stirring. The solution was then neutralized with 1N NaOH, 90 mg of $NaBH_4$ were added thereinto and the solution was kept at room temperature for 4 hours under stirring. The pH was brought to 5 with 2% HCl and maintained till the end of the effervescence. Finally, the pH was brought to 7 with 1N NaOH and the solution was ultrafiltrated with a 1,000 D cut-off membrane to give a solution containing a LMW-epiK5-N-sulfate having a mean molecular weight of 10,000 D. Its ^{13}C-NMR spectrum is shown in ANNEX 1.

Preparation of a LMW-epiK5-N,O-sulfate

(a) Oversulfation

The whole solution obtained in the above preparation, containing the LMW-epiK5-N-sulfate, was concentrated under reduced pressure and passed through an Amberlite® IR $120H^+$ column. The eluate was brought to pH 7 by addition of a solution of 15% tetrabutyl ammonium hydroxide (TBA) and the solution was let to stand one hour by concurrently

maintaining the pH 7 by addition of TBA, then the solution was concentrated under reduced pressure and freeze dried. The obtained solid, consisting of the tetrabutylammonium salt of LMW-epiK5-N-sulfate, was dissolved in 15 ml dimethylformamide (DMF) and the temperature was brought to 45°C under stirring. The solution thus obtained was then added under stirring with a solution of pyridine.SO_3 adduct (Pyr.SO_3), previously prepared by dissolving 1.43 g of Pyr.SO_3 in 15 ml of DMF. The reaction was left at 45°C for 18 hours. At the end of the reaction the solution was diluted with 1 volume of water and the final pH brought from 2.6 to 7 with 1N NaOH. The solution was submitted to an ultrafiltration in the presence of 2M NaCl with a 1,000 cut-off membrane. After drying, 330 mg of LMW-epiK5-amine-O-oversulfate were obtained.

(b) O-desulfation

The above sample of LMW-epiK5-amine-O-oversulfate was dissolved in 500 ml of deionized water and cooled to 4°C. The solution was passed through an Amberlite® IR 120 H^+ resin and the eluate (pH 2.92) was brought to pH 7 with a 5% aqueous solution of pyridine. The solution was concentrated under reduced pressure and freeze dried. The pyridine salt of the LMW-epiK5-amine-O-oversulfate thus obtained was dissolved in a 9/1 DMSO/CH_3OH solution and left at 65°C for 150 minutes under stirring. The solution was then diluted with 1 volume of water. The pH of the solution was 3.73 and was brought to 7 with 1N NaOH. The solution was finally ultrafiltrated with a 1,000 D membrane and 300 mg of partially O-desulfated LMW-epiK5-amine-O-oversulfate were obtained.

(c) Selective 6-O-sulfation

A solution of the obtained sample of partially O-desulfated LMW-epiK5-amine-O-oversulfate in 300 ml of water was cooled to 4°C and passed through an Amberlite® IR 120 H^+ column. The pH of the eluate was 3.18. The pH was brought to 7 with a 15% TBA solution and the sample was concentrated under reduced pressure and then freeze dried. The powder was dissolved in 15 ml of DMF and the obtained solution, cooled to 4°C, was added under stirring with a previously prepared solution of 955 mg of Pyr.SO_3. in 15 ml DMF. The mixture was left to stand at 4°C for 90 minutes under stirring and, at the end of the reaction, it was diluted with 1 volume of water. The pH was 2.47 and was brought to 7 by addition of 1N NaOH. The solution was finally ultrafiltrated with a 1,000 D cut off membrane in the presence of 2 M NaCl and 254 mg of partially O-desulfated, 6-O-sulfated LMW-epiK5-amine-O-oversulfate were recovered.

(d) N-Sulfation

The whole sample recovered at the end of step (c) was dissolved in 20 ml of water under stirring and the temperature was brought to 50°C. The obtained solution (pH 5.03) was brought to pH 11 by addition of 1.285 g of Na2CO3 and added stepwise with 1.285 g of PyrSO3 in powder. The reaction was kept at 50°C for 24 hours under stirring. The final pH was 7.62 and was brought to 7 by addition of 4% HCl. The sample was ultrafiltrated with a 1,000 D cut-off membrane and dried. A yield of 230 mg of LMW-epiK5-N,O-sulfate (called GL 2009/3) having an average molecular weight (MW) of 10,100 D was obtained. Its ^{13}C-NMR spectrum is attached herewith as ANNEX 2.

In order to obtain a LMW-epiK5-N,O-oversulfate having a mean molecular weight of 6,000, the product obtained at the end of step (d) was submitted to a nitrous acid depolymerization as follows:

An amount of 150 mg of the LMW-epiK5-N,O-sulfate obtained at the end of step (d) above was dissolved in 15 ml of water, cooled to 4°C and the pH was brought to 2.2 with a 2% HCl solution. A volume of 513 µl of a 0.029 M $NaNO_2$ solution, corresponding to 1.04 mg of sodium nitrite, was added and the reaction was kept at 4°C for 30 minutes under stirring. At the end, the pH was brought to 7 with 1N NaOH. Then 27 mg of $NaBH_4$ were added and the reaction was let to continue at room temperature for 4 hours. The pH was finally adjusted to 5 with a 2% solution of HCl till the end of the effervescence and then brought to 7 with 1N NaOH. The sample was ultrafiltrated with a 1,000 D cut-off membrane and dried. A yield of 110 mg of LMW-epiK5-N,O-sulfate having an average molecular weight (MW) of 6,000 (called GL 2143) was obtained. Its ^{13}C-NMR spectrum is attached herewith as ANNEX 3.

The interpretation of the ^{13}C-NMR spectra of both GL 2009/3 and GL 2143 was made by myself and the other inventor together while the Anti-Xa and Anti-IIa activities were determined by our licensee according to the method described in paragraph [0122] of the specification as published and, more specifically, as illustrated in my previous 3 March 2008 Declaration.

The chemical and biological properties of these products as well as of those of the product of Example 1 are summarized in Table 1 below.

Table 1

	N-sulfate	A-6-O-sulfate	A-3-O-sulfate	G-3-O-sulfate	I-2-O-sulfate	Sulfation degree	MW	Anti-Xa activity	Anti-IIa activity
GL 2009/3	95%	90%	40%	35%	20%	2.75	10,100	116 IU/mg	44 IU/mg
GL 2143	83%	82%	43%	32%	18%	2.32	6,000	157 IU/mg	40 IU/mg
Example 1	>95%	80%	50%	40%	20%	2.83	6,000	118 IU/mg	43 IU/mg

Notes: A = glucosamine units; G = glucuronic acid units; I = iduronic acid units.
Sulfate content, sulfation degree and molecular weight were calculated from the ^{13}C-NMR spectra.

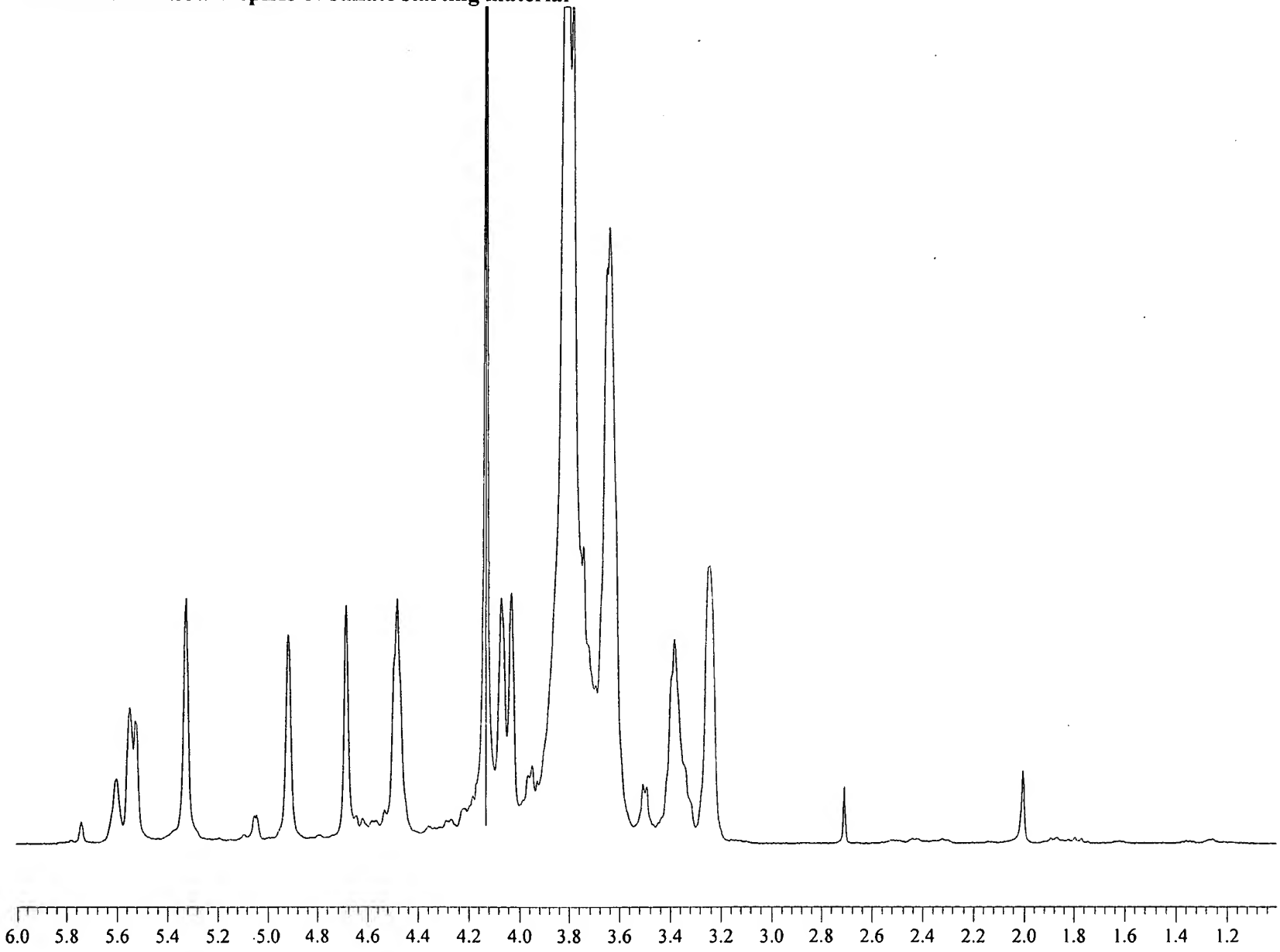
In two similar preparations, by depolymerization of GL 2009/3 two other epiK5-N,O-sulfates were obtained, GL 2144 (MW 6,200) and GL 2145 (MW not determined), having a sulfation degree of 2.26 and 2.19, respectively; and Anti-Xa/Anti-IIa activities (in IU/mg) of 153/34 and 157/40, respectively, practically overlapping those of GL 2143.

9. Since the product GL 2143 shows a sulfation pattern comparable to that of the product of Example 1 and still contains the predominant end unit (a'), while the few end units (a) introduced by the final depolymerization are irrelevant, the results summarized in Table 1 above show that said product has the characteristics given in claim 17 and an anticoagulant/antithrombotic activity completely different from that of the Oreste et al. reference but qualitatively similar (even though quantitatively better) to that of the product of Example 1. The same is also true for the products GL 2144 and GL 2145.
10. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that the making of willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the applications or any patent issuing thereon.

Dated: Milan September 1, 2009

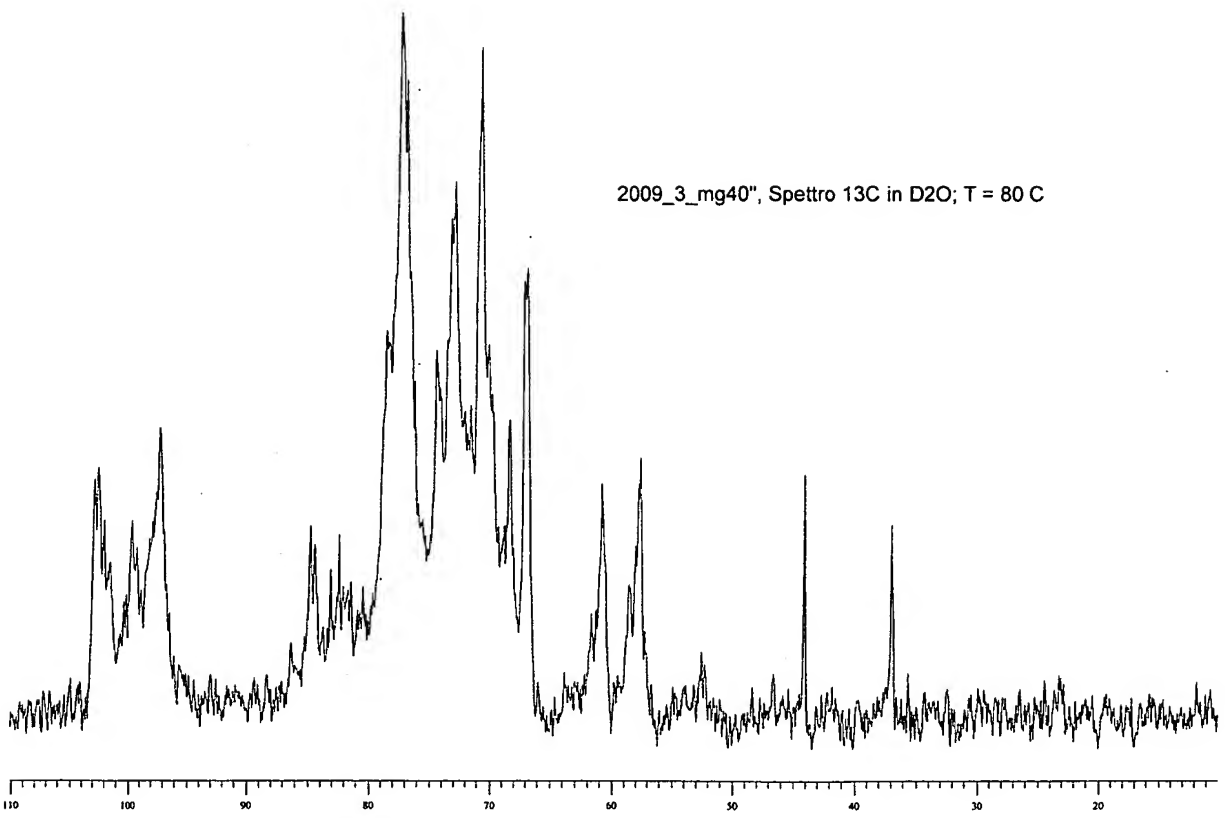
Pasqua ORESTE

^{13}C-NMR of the LMW-epiK5-N-sulfate starting material



ANNEX 2

^{13}C-NMR (D_2O; T = 80 °C) of GL 2009/3



Annex 3

^{13}C-NMR spectrum (D_2O; T = 80 °C) of GL 2143